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# ARTIFICIAL PARTHENOGENESIS

by

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OUTLINE FOR THESIS ON

ARTIFICIAL PARTHENOGENESIS.

# ARTIFICIAL PARTHENOGENESIS

I shall treat the subject of Artificial Parthenogenesis in four parts; first, a few introductory remarks; second, a brief outline of normal fertilization and development; third, the body of this thesis and my particular field, the subject of this paper; and fourth, a brief conclusion. Let it be stated here that I shall not deal with the philosophical, moral, social, or economic side of the question, but merely with experiments in the field and the facts established by them.

Much has been written on this subject and it has been of interest to man since 1667 when Goedart Bonnet demonstrated Artificial Parthenogenesis in the moth Orgyia. This new and surprising find led to other experiments. However, nothing of importance to science was added in this field for seventy-eight years. Then in 1745 Bonnet discovered virgin reproduction in the plant lice. A century passed idly away until Dzierzon in 1845 found that unfertilized eggs in the case of honey-bees gave rise to drones. Two years later, 1847, Bonsier working with silk worms startled science and silk manufacturers by stating that a female silk worm which had not been paired with a male, when put in the sun, produced eggs which hatched into caterpillars. This simple experiment which in the light of present knowledge seems highly improbable

and rather amusing, was discussed at length by men of intelligence, and caused quite a sensation in its day. However, these early chimeras and crude experiments were sufficient to keep up interest in the subject of eggs developing independently of sperm. Again, the spark of interest almost died, and it was not until fifty-two years later, 1899, that Vernon rekindled it. Since then a multitude of experiments right to the present day has taken place with surprising, confirmatory, puzzling and fascinating results. Some of the more recent experimenters are: Quojot (1905), Kellogg (1907), Escaillon (1916, 1917, and 1918), Cavazza (1924), Grandosi (1924), Loeb (1900-1913), Just (1922), R. S. Lillie (1914, 1916), and many others. Jacques Loeb has been one of the most persistent and careful workers in the field of Artificial Parthenogenesis, and so I consider it safe to base a considerable amount of this paper on his experiments and results. But first let us look at some of the earlier works that made it possible for Loeb to reach his conclusions. In the years 1896, 1897, Mead worked with the eggs of the marine worm Chaetopterus and found that normal eggs placed in sea water form a polar spindle when the nuclear wall has disappeared. The development of the egg, however, stops here unless a sperm is introduced. If this is done the two polar bodies are thrown off and cleavage sets in, after the junction of the pronuclei. Further experiment showed that eggs treated with a solution containing one-half of one percent sodium chloride

throw off the polar bodies, and the first cleavage takes place. Later the characteristic protrusion of the yolk lobe appears. He could go no further in the development of the embryo, but a start had been made in the right direction towards the artificial development of eggs.

At about the same time Hertwig working with sea urchin eggs found that if they were treated with strichnine, radiations around the nucleus were formed in preparation for division.

T. H. Morgan in 1899 treated sea urchin eggs with solutions of sodium and magnesium chloride and came to the following conclusions: first, that eggs thus treated cleaved though not having been fertilized; secondly, that asters were formed, though artificial, and took part in the cleavage; and, thirdly, that the formation and development of a bipolar spindle took place with centrosomes at its poles. He also found that cleavage was not quite normal in these larvae and he could induce no development beyond the blastula stage. His results in 1900 can be summarized in the following table in which a five percent solution of magnesium chloride was used:

| Length of time eggs treated | 5 min. | 10 min. | 20 min. | 30 min. | 60 min. |
|---|---|---|---|---|---|
| Fraction of eggs that developed | 1/4 | 1/3 | most | 1/2 | none |

In the nineties of the last century Loeb worked with echinoderm eggs and found normal fertilization and development to be as I shall briefly outline it. The eggs of Arbacia or Strongylocentrotus purpuratus were cut from the female and placed in a dish of salt water. A solution of salt water and sperm was then introduced. The sperm agglutinated to the egg and swam around it. It has also been found that this agglutination and swarming take place, if sperm are introduced to sea water in which eggs have formerly been at one time. Many explanations have been offered for this characteristic action on the part of the sperm, but as yet nothing definite is known. R. S. Lillie, who has worked for a long time with this problem and that of artificial parthenogenesis, believes that a substance diffuses from the egg into the surrounding sea water and that it is this that attracts the sperm. Such a substance as this has not been detected as yet in egg water, even with the most careful experiments. Loeb seems also to be in favor of this diffusion theory.

Another theory is that there is a difference in electrical potential between the egg and sperm which is such that it draws the sperm to the egg. It has also been found that if a positive and negative pole is put into a solution of sperm and sea water, the sperm will gather at these poles and also agglutinate in masses throughout the solution. It appears, to me at least, that the diffusion theory is the more probable, since the sperm agglutinate in egg water which would seem to

mean that something had been left behind by the eggs, when they were drawn off. As I have mentioned above, however, nothing definite has been shown in either case.

Passing over to the initial development of the eggs we find that the surface layer of the egg is somewhat sticky, for the sperm adheres to it. Eventually, a sperm pierces this zona pellucida. In some cases the egg seems to shrink inside this clear substance and send a protoplasmic thread out to the sperm to draw it in. Again, the sperm has to reach the surface of the egg with no help from the latter. The vitelline membrane is now formed very quickly. This is done by small droplets of membrane substance forming on the egg surface. They grow rapidly in size, and a little later fuse over the entire surface of the egg, forming a solid wall. This membrane formation is exclusive to the other sperm and they soon cease their activity and die. Occasionally polyspermy takes place, when two sperm--rarely more than two--reach the surface of the egg at the same time. After fertilization several membranes are recognizable in the egg. There are the chorion directly surrounding the cytoplasm, the vitelline membrane, the fertilization membrane and the zona pellucida over this. This is in accordance with the observations of J. S. Carter.

Let us take now the case of a single sperm entering the egg, for this is the normal procedure in the formation of the zygote. The sperm on piercing the chorion loses its

tail, and the head which is a nucleus plus the middle portion of the sperm advances through the cytoplasm towards the egg nucleus. Much has been speculated concerning this middle portion of the sperm. It has been thought to be the sperm polar body brought to the egg to aid astral formation. It has also been believed that it carried to the egg some protective substance against cytolysis and disintegration after fertilization, because it has been noticed that eggs parthenogenetically treated will cytolize after membrane formation. This, however, will be taken up in more detail later in this paper. Whether this mid-section of the sperm really serves these purposes or not has not convincingly been shown. The sperm nucleus now fuses with that of the egg. A little later asters and astral rays are formed at opposite poles of the egg. The nucleus now at about the center of the egg divides and the chromosomes begin to move towards the two poles, a similar number going to each pole. The first cleavage now sets in from the surface of the egg, and G. S. Carter has noticed that the chorion and vitelline membrane follows this line of cleavage. The first two cleavages are vertical and the third is horizontal. It is hardly necessary to say that two nuclei, one at each pole, have been formed in the original egg and that cleavage takes place between these. The first cleavage is followed shortly by the second, which is very similar except that

there is no introduction of material from the outside. Cleavage takes place regularly now, the cells becoming smaller, but more numerous through the four, eight, sixteen, thirty-two, sixty-four, one hundred and twenty-eight, etc., celled stages, till the blastula, a hollow sphere of cells, is formed. This develops cilia and swims about inside the membrane surface, or may be free in the salt water. Development continues and an invagination is formed in the spherical surface of the egg. This is at first slight, but later becomes very deep. At about this time mesenchyme cells are budded off from the infolding portion of the sphere which becomes the entoderm in contrast to that which is not infolded and becomes the ectoderm. These mesenchyme cells later form the spicules of calcium carbonate that make up the skeleton of the adult, and are first seen in the pluteus stage. The infolding entoderm develops the tripartite gut of the pluteus with the blastopore at one end and the mouth at the other, which is formed by the anterior end of the gut reaching the surface of the embryo; joining with it and disintegration taking place inside the circumference of the connecting layers, leaving a round hole to the outside world. The normal pluteus seeks the surface of the water. Those injured during development or improperly fertilized usually sink to the bottom where they either disintegrate, become food for bottom living forms, or die from bacterial attack.

It should be mentioned here that the oxidation consumption of the egg increases from four to six times after fertilization. This means that very rapid and important metabolic changes are taking place in the egg. Loeb, therefore, believes that it is against these metabolic changes and greatly increased rate of oxidation that the sperm protects the egg and also causes it to develop. More than this, the sperm brings to the egg the male chromosomes with their characters. This produces the diploid condition of the cells with the exception of the sex cells in the adult. Loeb has noticed that fertilization in Asterias does not cause an increase in oxygen consumption. Also he has been able to hinder nuclear division and subsequent cleavage by placing fertilized eggs in a dilute solution of sea water plus KGN; by reducing the temperature to $0^{\circ}$ C; removing oxygen from the water in which they were placed; or by keeping them in a stream of hydrogen. On returning to normal sea water, nuclear and cell division continued normally. Furthermore, sea urchin eggs can be fertilized by the sperm of other species; viz., starfish, brittle stars, holothurians, crinoids, and even molluscs; and yet develop into normal larvae. In this way, they differ from those of Asterias which cannot be crossed with sperm of other species.

Having followed the development of the egg from fertilization to the pluteus stage, let us now look at the constituents of the egg itself. Not a great deal is known about

the chemical composition of the egg, because it is very complex. Around the cytoplasm there is a membrane which is probably made up of lipoids, though it does not react completely to tests for lipoids. There are lysins present and complex protein material. This membrane is semi-permeable as is shown by the fact that weak bases and acids affect the egg much more quickly and effectively than do strong ones. There is a large amount of water in the egg which is taken in after fertilization. This is demonstrated by placing the eggs in hypertonic sea water, where they shrink in size because of giving up this water to the surrounding solution which has less concentration than the egg. This lost water will be taken up again, if the eggs are peplaced in normal sea water; if not they disintegrate. There is also a sudden increase in the acid content of the egg following fertilization. The egg seems to consist mainly of protoplasm of which we know very little, and of yolk or food material. As regards the sperm, we know that it is practically a naked nucleus, containing darkly staining chromosomes. The structure of the egg with its nucleolus containing chromosomal material, nucleus surrounded by cytoplasm containing yolk material, often salt crystals and protoplasm and about which stretches the semi-permeable membrane is so well known that we need not discuss it further here.

In certain insects and some crustacea, there is a tendency towards parthenogenesis, and before progressing to the main theme of this paper it might be well to glance at some of these for a moment. Solenaria, a butterfly, lays parthenogenetic eggs which develop into quite normal adults in a perfectly normal way. According to von Siebolt Psyche helix does likewise. Dzierzon has observed that the parthenogenetically laid eggs of Bombus develop into males and queens, while those normally fertilized become females. The idea that parthenogenetic eggs should develop females like the mother does not always hold as von Siebolt and Leuckart have shown. They found that the parthenogenetic eggs of the Psychidea and Solenobia developed into females while in bees they became males only. Again in crustacea, such as Apus and Artemia the unfertilized eggs became females only.

So far we have dealt with the early rather confused observations and experiments of various workers with the egg. We have seen how the fertilized egg develops normally, though why it does so is still a mystery and we have noted that certain eggs have a natural tendency toward parthenogenesis. Now let us see what man's interference in this complicated mechanism of development has been, and what, if anything, he has accomplished.

Jacques Loeb, having worked with and studied the normal development of fertilized eggs, began to wonder if he could

not cause eggs to develop without the help of sperm. He was not the first to ask this question, but he has worked as long as anyone else and has obtained many interesting results. His first clumsy attempts for a method have developed today to a high technique, and his carefully controlled and rigorously accurate accounts of his experiments form an excellent basis on which to build this thesis. Loeb is a mechanist, due largely to the results of his own experiments and those of others. He believes that if we could just find out the chemical composition of the egg and sperm we could in all probability set the mechanism of life going ourselves. I do not believe it is putting it too strongly to say that he considers life a complicated series of chemical stimuli and reactions. This is the impression one gains from reading his works. Loeb noticed that one of the first observable changes in the egg was the formation of the fertilization membrane after the introduction of sperm. He, therefore, tried to produce this membrane chemically. In 1892 he put some virgin eggs of Arbacia into a hypertonic solution of sea water (100 cc. sea water + 2yNaCl). When these eggs were replaced in normal sea water they divided. He experimented further and found that these eggs disintegrated if left in the hypertonic solution too long, that they lost water in the solution which they regained in normal sea water, and finally that these eggs, peculiarly

enough, often broke up suddenly into many nuclei and cells in sea water, showing that a nuclear and cell division had taken place in the hypertonic solution though it was not visible in the egg while it was in that solution.

Van't Hoff, having worked for years to discover the chemical composition of sea water, finally reached the following formula: 100 mol's.NaCl. 2.2 mol's KCl, 1.5 mol's $CaCl_2$, 7.8 mol's $MgCl_2$, 3.8 mol's $MgSO_4$, where the $^{c}Ho$ was about $10^{-7}$ N or neutral. To 50 cc of this Loeb added 0.0, 0.1, 0.2, 0.4 and 0.8 cc of N/100 K O H. He found that no eggs developed in the first solution beyond the four to eight celled stage. In the second a few eggs developed a little further. In the third sixty percent reached the blastula stage. In the fourth and fifth solutions all the eggs became larvae. It would seem, therefore, that a slightly alkaline solution is necessary for the early development of the egg. Later it will develop in a neutral solution.

Salt solutions of sodium, lithium and rhubidium cause muscle fibers to contract rhythmically, while calcium and strontium salt solutions prevent this. The former salts were injurious to the muscle fibers, and the latter seemed to have a preservatory effect. Loeb got the idea from this of changing the content of the sea water which he believed prevented unfertilized eggs from developing.

He found that treatment with weak acids and bases initiated development. The eggs must, however, be placed in a hypertonic solution of sea water for a while before being put into normal sea water. If this is done normal division takes place, and normal larvae are eventually formed. He found that if eggs were left in monobasic fatty acids--formic, acetic, proponic, volerianic--all caused segmentation and membrane formation. Also that solutions of a non-electrolitic character viz., sugar and urea with relatively low molecular concentration and an osmotic pressure 40% that of sea water is sufficient alone to cause sea urchin eggs to segment. Bataillon (1900) and Loeb at about the same time, came to the conclusion that the increase of osmotic pressure of the medium into which eggs had been put though small was the factor that caused segmentation. Loeb went further than this and stated that no matter how membrane formation was started in a solution of increased osmotic pressure except in solutions containing poisonous salts, such as copper, eggs would develop. He believes that membrane formation is the deciding condition for development. He found that normal larvae could be obtained by first treating them from 1.5-2.5 minutes with lye, fatty acids, sugar solutions, saponin, bile salts, hydrocarbons, etc., then with a hypertonic solution of sea water and finally placing them in normal sea water.

The reason he gives for the use of the second treatment, or that with the hypertonic sea water, is that membrane formation initiates development and also a rapid series of injurious oxidations takes place in the egg, as we have seen after fertilization, the rate of oxygen consumption is raised four to six times. This leaves the egg in a very sickly condition which can only be restored to normal by treatment with a hypertonic solution, or by slowing up these oxidations by some other means, such as the withdrawal of oxygen for a time, treatment with K C N, or by keeping the egg for a greater or less period, depending on the kind of egg used, in a stream of hydrogen, or by chilling the eggs to 0° at which point their oxidation rate becomes about zero. This slowing up of oxidation gives the egg time to recover from its artificial treatment and can then proceed to develop normally when returned to normal sea water. In normal fertilization, the entrance of the sperm into the egg prevents disintegration after membrane formation. Just how this is done we do not know. The injurious effects of normal membrane formation are prevented by the sperm, but in artificial membrane formation as we have seen there must be a second treatment that suppresses the rapid rate of oxidation. In the case of the starfish this is not so essential because after fertilization the oxidation consumption rate is hardly noticeably increased.

Hypertonic solutions have their limit, however, for they must be within 50 cc. of sea water + 5-12 cc. 2.5 M NaCl. If they are stronger than this they injure the egg after membrane formation. If they are weaker, then the eggs do not develop. The temperature also is important and must be from 16.5° -17.5° C. to obtain the best results. The effect of the hypertonic solution is nullified by K C N, also it is only effective if there is a sufficient amount of free oxygen present. Very few eggs develop if they are treated with a hypertonic solution before membrane formation. If, however, the treatment is after membrane formation most of them develop. This is because the hypertonic solution cuts down the rate of oxidation after membrane formation which as we have seen is increased from four to six times.

For a moment let us review Loeb's work and draw what conclusions we can from it. We have seen above that he based the beginning of development on membrane formation which can be initiated by, (1), treatment with a fatty acid, or by a monobasic base; (2), treatment with anesthetics, chloroform, benzol, toluol, xylol, etc.; (3), treatment with such specifically parthenogenetic agents as saponin, salonin and bile salts. Later as we shall see he used injections of blood serum and eventually

Hypertonic solutions have their limits, however, for they must be within 50 cc. of sea water + 8-12 cc. 2½ n NaCl. If they are stronger than this they injure the egg after membrane formation. If they are weaker, then the eggs do not develop. The temperature also is important and must be from 15.5° -17.5° C. to obtain the best results. The effect of the hypertonic solution is nullified by K C N, also it is only effective if there is a sufficient amount of free oxygen present. Very few eggs develop if they are treated with a hypertonic solution before membrane formation. If, however, the treatment is after membrane formation most of them develop. This is because the hypertonic solution cuts down the rate of oxidation after membrane formation which as we have seen is increased from four to six times.

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form membranes in as alkaline solutions as 50 cc. sea water + 5 cc. N/10 NaOH but not in 50 cc. sea water + 6 cc. N/10 NaOH. Even after membrane formation has been initiated the following divisions of the egg are irregular and the larvae are likely to be abnormal. This is largely due to the fact that the eggs of different females and even of the same female differ in the length of time that they must be exposed to the hypertonic solution to gain the best results. In Loeb's experiment of 1913, that has just been related, and in that of Just (1921) where membranes could be formed, if the eggs were later sperm fertilized in Arbacia, we find that this is not the case with Sphaerechinus, no membranes being formed in either case. Eggs without membranes, however, can be fertilized in water that is slightly acid where the pH is 5-6.5, or can be caused to develop parthenogenetically in water at least as acid as pH 5.0. The radius of the fertilization membrane, i.e., the thickness of the osmotic layer is reduced in acid water and the mechanism of membrane formation is destroyed when the acidity is too great. We see then that there is a fundamental difference between these two closely related echinoderms. In one, Arbacia, it is comparatively easy to cause the eggs to develop parthenogenetically and get normal larvae. In the other, Sphaerechinus, it is far more difficult and abnormal development usually results.

Loeb believes that surrounding the egg is an emulsion which protects the egg against outside influences. In order that development shall start, this must be broken down. This is done in normal fertilization by the sperm puncturing the egg and entering it. It is this emulsion also which prevents the fertilization of the egg by foreign sperm in many cases. When initiation of development is started artificially, this membrane must be dissolved or mechanically broken. Chemicals such as salts, acids, bases, etc., can only diffuse into the egg after this emulsion is broken down. Mechanical breaking down of this emulsion to start development is either by shaking, stroking or puncturing the egg with a needle. Loeb's method for treating eggs chemically to start development is as follows: eggs are put in a solution of 50 cc. sea water containing a certain amount of N/10 butyric acid 2.6 cc. in the case of Strongylocentrotus purpuratus in California 2.0 cc. in the case of Arbacia at Woods Hole for from two to four minutes. Ten to fifteen minutes later, the eggs are put into hypertonic sea water (50 cc. sea water + 8 cc. 2 1/2 NaCl, Ringer solution or cane suger solution) in which they remain at 15$^{\circ}$ C. from thirty-five to sixty minutes in the case of S. purpuratus, and from seventeen and one-half to twenty-two and one-half in the case of Arbacia. If the eggs are now transferred to

Loeb believes that surrounding the egg is an emulsion which protects the egg against outside influences. In order that development shall start, this must be broken down. This is done in normal fertilization by the sperm penetrating the egg and entering it. It is this emulsion also which prevents the fertilization of the egg by foreign sperm in many cases. When initiation of development is started artificially, this membrane must be dissolved or mechanically broken. Chemicals such as salts, acids, bases, etc., can only diffuse into the egg after this emulsion is broken down. Mechanical breaking down of this emulsion to start development is either by shaking, shocking or puncturing the egg with a needle. Loeb's method for treating eggs chemically to start development is as follows: eggs are put in a solution of 50 cc. sea water containing a certain amount of N/10 Butyric acid 2.5 cc. in the case of *Strongylocentrotus purpuratus* in California 2.0 cc. in the case of *Arbacia* at Woods Hole for from two to four minutes. Ten to fifteen minutes later, the eggs are put into hypertonic sea water (50 cc. sea water + 8 cc. 2 1/2 NaCl, sugar solution or cane sugar solution) in which they remain at 15° C. from thirty-five to sixty minutes in the case of *S. purpuratus*, and from seventeen and one-half to twenty-two and one-half in the case of *Arbacia*. If the eggs are now transferred to normal sea water, they will develop into normal larvae. An over-exposure to the hypertonic solution brings about abnormal development. This is merely a characteristic method that Loeb used to cause membrane formation. Many other substances than butyric acid can be used; viz., fatty acids, saponin, solonin, or bile salts, lipoid solvents as benzol, toluol, xylol, amylene, chloroform, aldehyde, ether, alcohols, etc., bases hypertonic or hypotonic solution, rise in temperature and certain salts as barium chloride, strontium chloride, etc.

Another method of causing membrane formation that has been mentioned already is that of using blood serum. Eggs, however, must have their susceptibility to foreign blood serum increased, because it has been found that extracts from the tissues of the same species as the egg does not bring about membrane formation. This can be done by putting the eggs in a solution of strontium chloride or barium chloride in sea water for from five to ten minutes.

Loeb found that the blood serum of Dendractema diluted 1 cc. per 50 cc. - 200 cc. sea water caused normal development in about twenty percent of the females used if the eggs were treated with hypertonic sea water afterwards. Membrane formation never takes place in the acid when the eggs are subject to it, but later in the normal sea water.

It is different with sipunculid blood serum, however, for in this case the membrane is formed in the solution. The blood of rabbits, pigs, dogs and oxen has been found to be effective if rendered isotonic by sea water with the addition of 2.5 m NaCl solution (1cc. of the 2.5 m in NaCl solution was added to 6.5 cc. of the serum). As has been stated above, eggs of sea urchins are immune to the blood of their own species. This is because the blood of the same species does not diffuse into its own cells. Some eggs, as those of S. purpuratus will develop merely by being subjected to hypertonic sea water. The conclusion that it seems safe to draw from all these experiments is that the spermatozoön helps development only by altering the surface of the egg in a way comparable to cytolysis of the cortical layer.

So far we have dealt principally with Loeb's work in the field of artificial parthenogenesis. Now let us turn to some of that by other experimenters, keeping Loeb's work in mind.

Just in 1922 using the eggs of Arbacia says that success in obtaining plutei lies in the proportion of salt solution in the hypertonic solution and on the length of exposure. The treatment with butyric acid he thinks to be unnecessary. Furthermore, the eggs of different females differ widely as to their reactions to treatment. He found that eggs lift off their membranes in a salt solution of

20, 22, 24 parts of 2.5m.NaCl or K Cl + 80, 78, 76 parts of sea water. This happens in from fifteen seconds to five or ten minutes, depending on the strength of the solution. Since he believes that the first treatment with acid is unnecessary, he concludes that the second treatment with the hypertonic solution is superfluous also. It will be remembered that Loeb's conclusions were quite the reverse of this.

Wilson (1901) obtained larvae from eggs by subjecting them to a magnesium solution of equal parts of sea water and of a twelve percent solution of magnesium chloride. With these eggs of toxopneustes, he got gastrulae, blastulae, and in many cases plutei, but these last were never quite normal.

Herlant (1918, 1919) working with the eggs of Paracentrotus lividus found that treating the eggs with acid merely disolved the chorion and that one hour and forty-five minutes later the nuclear wall also disolved. Then an irradiation that extends outward and a concentration of the cytoplasm takes place. The monaster which is formed loses its rays after about two hours and forty minutes. Boveri(1903)and Painter(1918), working along the same general lines as Herlant, noticed the irregular outline of the egg and an abortive attempt to divide at this point. This series of stages, concentration of cytoplasm, aster

formation and an attempt to divide may be gone through five or six times with a constant enlargement of the nucleus. After these attempts to divide, the egg begins to disintegrate. If eggs are treated by Loeb's method (which has been discussed) several asters may be formed whose rays extend and touch one another, forming a multi polar figure. Usually not more than three asters are formed and in these cases the resultant larvae are not normal. It is plain then that too many asters are injurious to development. In the case of normal fertilization, asters are formed when the eggs are treated with a hypertonic solution. But the normal machinery of segmentation is interfered with in proportion to the number of asters formed, i.e., if two asters are formed, there results a normal two-celled embryo; if three asters are present, there is found a three-celled embryo which is not normal and an abnormal larva results.

Loeb has shown that an alkaline solution is more efficacious in bringing about normal development. Herlant and R. S. Lillie found that by suppressing aster formation and cleavage for a time with K C N or, ether, alcohol or chloral hydrate, more nearly normal larvae could be obtained.

Herlant experimenting with the permeability of the fertilization membrane found that eggs activated by sperm or butyric acid and transferred every five minutes to a hypertonic solution of sea water in which they were left

for from forty-five to sixty minutes showed that the membrane is permeable. It later becomes semi-permeable and finally impermeable. This was shown by the fact that only those eggs were plasmalized that had been treated within forty to fifty minutes by a hypertonic solution after being activated.

Morgan[1] in speaking of the usefulness of the hypertonic solution believes that it is only active if the salt penetrates the egg. He says "It is the presence of the salt in the egg and not the dehydration of the egg that is the significant factor in artificial parthenogenesis."

It has been found that sodium chloride and potassium chloride augment the permeability of the egg, while calcium chloride and magnesium chloride diminish it. Herlant has shown that OH ions increase permeability and that H ions decrease it, at the same time weakening aster formation. Anesthetics have a similar effect on the egg as the H ions. He says further that it is the salts that penetrate the egg that cause cytaster formation. Also that protoplasm is a reversible gel in which asters are formed at the time that the egg becomes permeable to the salt water solution. This is somewhat similar to Morgan's conclusion that the salts entering the egg are the factors bringing about artificial development.

[1] T. H. Morgan, Experimental Embryology, page 554.

Fry (1925) disagrees with Herlant in regard to aster formation and subsequent development. He says that the most nearly normal embryos are produced in those eggs whose mitotic figure arises by the division of the aster near the nucleus and not as Herlant supposed by a combination of the central and a peripheral aster. Fry used the most recent and effective method of treating eggs which was worked out by Just (1919). It is a combination of butyric acid solution and sea water (2cc. 1/10 normal butyric acid plus 50cc. sea water). The eggs are put in this solution for about thirty-five minutes and then transferred to sea water where the membranes are lifted off after twenty-five minutes. This is followed by a treatment of a hypertonic solution of sodium chloride (5cc 2.5m NaCl + 50 cc. sea water). He found that eggs thus treated formed a nuclear aster and a cytaster but did not develop into normal larvae. There seems to be a slight difference between these two asters, because he noticed that the cytasters rarely divide while the nuclear aster does so readily, is near the nucleus and is larger than the cytaster. Nevertheless, the nuclear aster can combine with the cytaster, forming a multipolar mitotic figure in which there is a migration of chromosomes to all the poles. Because of the more regular distribution of the chromosomes to the poles in the case of a single aster being formed, a more normal embryo is

formed. Fry's conclusion seems to be logical and possibly more accurate than Herlant's, since he used the most modern methods of treatment, while Herlant worked with older, less effective ones.

I have dealt primarily with the eggs of sea urchins in relating these experiments, but a great deal of interesting work has been done on other forms such as molluscs, annelids, starfish, frogs, etc. It is to these that I wish to turn now.

Artificial parthenogenesis in starfish is quite a different matter from that in sea urchins. The methods used to start development in sea urchins of using Na Cl or butyric acid do not work with starfish, but $CO_2$ in the sea water and mechanical agitation bring about good results. These last two methods do not work with sea urchins. The reason for this is that when the starfish eggs are laid they are immature and must throw off the polar bodies before fertilization or development can take place. Having done this, they are ready to be worked with. For this reason also oxidation is not increased when fertilization takes place; as we have seen, it rises in the case of the sea urchin egg. When starfish eggs are placed in sea water, the first polar body is given off. They are then put into a solution of sea water plus $CO_2$ for about an hour. This is followed by returning them to normal sea water again. In eggs thus treated the second polar body is not usually given off

and segmentation sets in. The eggs gastrulate, swim about, and the auricular stage is reached in about five days. With this method only those eggs that have given off the first polar body segment. Those eggs whose nuclei remain intact when they are put in the sea water containing $CO_2$ never develop further.

Mathews (1901) experimenting with the eggs of Asterias forbesii allowed them to ripen until both the polar bodies has been given off. He then put them in a test tube half full of sea water and shook them violently. After this shaking, he replaced them in dishes containing sea water and left them for from four to six hours. At the end of this time slight agitation of the dishes or transference from one dish to another by a pipet caused development to start and gastrulation had taken place by morning. Mathews' notes in regard to this experiment are totally unsatisfactory. He makes no mention, for example, whether there were any normal larvae formed by this method, nor does he have anything to say as to whether cleavage was normal or not. Probably very few if any normal larvae resulted from this treatment. We do know, however, from his notes that from less than one percent to fifty percent developed into swimming blastulae. Delage working with the same material and method, never obtained more than fifty percent blastulation and he only got thirty swimming

gastrulae from four thousand eggs. From another batch of two thousand five hundred eggs, he was able to produce only seventy-five blastulae and gastrulae, and in many cases even less than this.

Buckner (1911) has done some very careful and observant work with the eggs of Asterias gracialis. He treated them with a combination of sea water and $CO_2$. On returning these to normal sea water, the first spindle was formed. Slowly the first polar body spindle divides. This leads to the formation of an outer and inner group of polar chromosomes. The outer group may or may not be extended in a polar body, if not, it frequently becomes vesiculated and comes to lie in a flattened mass of cytoplasm that scarcely protrudes from the egg surface. The inner group of chromosomes becomes vesiculated also and a second polar spindle forms on which the resolved chromosomes now collect. Here typical division takes place and the daughter halves move to the poles. This is the anophase stage. Both sets soon become vesiculated and these vesicles unite to form larger ones. Finally, in typical cases two nuclei develop within the egg cytoplasm. These two unite and sink deeper into the egg where they become the segmentation nucleus. In this way, as Delage supposed, the second polar body is suppressed. Its nucleus unites with the egg nucleus to form the nucleus of segmentation. Each blastomere has

thirty-six chromosomes which is the normal diploid number. It is just the reverse of this in the case of the sea urchin in which the cells are haploid.

Tennet and Hogue worked with the eggs of *Asterias forbesii* and *Asterias vulgaris*. They treated these eggs with $CO_2$, but had great difficulty in determining the exact number of chromosomes, because they seemed to adhere closely to one another. They finally reached the conclusion that some had eighteen chromosomes while others had only nine. Comparing this to Buckner's results we should say that some of these were haploid, while others were only a half of this.

R. S. Lillie(1914, 1916) exposed the eggs of starfish to butyric acid for from six to ten minutes. When these eggs were returned to normal sea water they cleaved more or less regularly, and eighty to ninety percent formed normal larvae. He found that exposure to high temperatures brought about similar results and that for each rise in temperature a minimal exposure will induce membrane formation. An exposure of two minutes at 32° C is sufficient to bring this about. A longer exposure of eight minutes at the same temperature causes more complete activation, but an exposure of twelve minutes is too long and the eggs fail to develop. This shows that a definite quantity of reaction-product is formed. The optimum exposure for the best

results at 31° C. he found to be thirty minutes; at 32° C. seven to eight minutes; at 34° C. three minutes, and at 36° C. one minute. He makes the suggestion that the viscosity of the egg may permit the combination of constituents which in their early stages are kept apart through their inability to diffuse, and concludes, therefore, that it is the rate of diffusion that determines the result. As we have seen in the case of the sea urchin egg, bases and acids activate it, but Lillie believes that it is the rate of penetration of these into the egg that is the chief factor in determining the reaction.

Lillie was much impressed in his experiments with the analogy of nerve action when stimulated and that of the sea urchin egg when activated. The nerve, of course, acts at once while the sea urchin egg goes through a rhythmic series of cleavages. The critical event in nerve excitation is a temporary change in electrical polarization. One of the first changes in a fertilized or artificially treated egg is an initial increase in permeability. This change should be accompanied by an electrical polarization of the surface membrane. Miss Hyde (1904) working with the egg of Fundulus found this to be true. These eggs after fertilization or artificial treatment depolarize for the first fifteen minutes, and

then return to their original state of polarization. This takes place with an increase in permeability.

Lillie placed eggs in anesthetics, ether, ethel urethane, chloral hydrate, chloratone and various alcohols. After this treatment, they were subjected to a hypertonic solution of sea water for thirty minutes and later put into normal sea water. Many larvae were obtained in this way. This initial depolarization caused by an increase in membrane permeability is probably the critical or determinative event in fertilization as well as in stimulation, Lillie believes. Cytolisis results if the egg is not returned to its originally polarized condition of semi-permeability within a certain period at 20° C. after such treatment. The return to the semi-permeability of the membrane with the correlative electrical polarization is favored by treatment with cold, cyanide anesthetics, hypertonic sea water, whose general action is permeability-decreasing, or anti-cytolytic. He further found in 1914 that the formation of fertilization membranes and the initiation of cleavage are prevented by anesthetics when the parthenogenetic agent is a neutral salt, but not so when it is a fatty acid. He explains this by stating that the anesthetic renders the plasma membrane less permeable in the case of salts, but that the fatty acid which enters the egg by virtue of its lipoid solubility at all times is

not prevented from acting. Experimenting with the effects of ultra-violet rays on the eggs of Arbacia, he found that the eggs responded more readily to treatment with hypertonic sea water afterwards. Similar effects after violet-ray treatment resulted if the eggs were shaken for several seconds or minutes. Too much shaking, however, injures them and the results are negative. Lillie and Cattell (1925) obtained deformed specimens by subjecting eggs to strong electric currents.

Loeb has also done considerable work with starfish eggs. He claims that artificial substances are analogous though perhaps not similar to those found in the sperm. He found that the eggs of Asterias formed membranes, if placed in a solution of 50 cc. sea water plus 1 cc. amylene. If the eggs are left too long in this solution, they will disintegrate. Eggs placed in 50cc. sea water to which 5cc. N/10 acetic acid has been added form membranes after two minutes. In the case of starfish eggs, the treatment with hypertonic sea water of K C N is unnecessary, but they develop into larvae when returned to normal sea water. All sperm fertilized eggs develop normally into larvae. Only ten percent of those eggs treated with 6cc. N/10 butyric acid + 50cc. sea water developed into larvae. The rest disintegrated. When eggs are shaken they develop a fertilization membrane. This is due, Loeb believes, to the breaking of the emulsion on the outside of the egg which allows for the absorption of the water.

Loeb found that starfish eggs die just as quickly in sterilized flasks as in those that he had befouled or contaminated with bacteria and infusoria. This shows that it is not bacteria and infusoria that bring about death to unfertilized eggs, but the intrinsic factors of disintegration in the egg. If eggs are fertilized, they do not die. The lack of oxygen will prevent the development of the unfertilized egg. The same result is obtained if five or six drops of 1/10 percent KCN is added to 50 cc. sea water containing eggs. The act of fertilization changes the egg from an anaerobe to an aerobe and renders the egg immune to the destructive factors of membrane formation and oxidation which hasten the death of the unfertilized egg. He concludes that it is the causation of development and not the action of one of the two factors alone which saves the life of the egg.

The eggs of molluscs will develop when treated artificially like sea urchin or annelid eggs, but do not form any membranes and they must have oxygen. Wastneys obtained normal results by treating mollusc eggs with ox blood serum. The eggs had to be sensitized to the effect of the serum so they were placed first in a solution of strontium chloride for two minutes. Next they were put in a solution of ox blood serum rendered isotonic by sea water diluted with an equal part of m/2 solution

of $NaCl + CaCl_2 + KCl$ for a period of five minutes. They were now freed from all traces of the ox blood serum by several washings with Ringer solution. Finally, they were placed in a hypertonic solution of sea water (50cc. sea water + 8cc. 2 1/2 m NaCl). Control experiments showed that the ox blood serum was essential to the success of the experiment.

Much interesting work has been done with the eggs of annelids, starting rather unsuccessfully in 1898 with Mead using Chaetopterus eggs. Other workers with annelid eggs have been Loeb (1901), Lillie (1902, 1906), Bullot (1904), Fischer (1902), Scott (1906), Lefevre (1907), Allyn (1912), and Just (1915).

Lefevre (1901) obtained irregular cleavage in eggs treated with $Mg\ Cl_2$, NaCl, $Ca\ (NO_3)_2$, K Cl and weak solutions of strong or weak acids. He used for his experiments the eggs of Thalassema and found that there was a great variability in his results. For example, he put eggs in a solution of 15 cc. m/10 HCl + 88 cc. sea water for five minutes and sixty percent of them became larvae. Then he took more eggs from the same female, put them in the same solution but left them there for six minutes, and only five percent became larvae. When the optimum conditions are present, the eggs cleave normally and can not be distinguished from the sperm fertilized eggs. Perfectly normal development

takes place. Normal gastrulation takes place, a normal trochophore is formed, polar spindles are present and polar bodies are given off in the usual way. It is not necessary that either or one of these polar bodies be given off to obtain normal results. The first indication of cleavage is that two small asters are formed on opposite sides of the nucleus. It has not been ascertained how many chromosomes there are in these cases. It would be expected that the number would be diploid in the first case while there would be forty-eight chromosomes in the second. In the first case two nuclei are formed and these fuse. In the second instance two nuclei are present but they do not fuse.

Most of the earlier work on annelid eggs turned out rather badly. It seemed quite a difficult problem to get good results as we shall see. Fischer (1902) treated the eggs of Nereis with a solution of 80 cc sea water + 20 cc KCl 2 1/2 m. He obtained nothing but monstrosities. Lillie (1902, 1906) treated eggs of Chaetopterus with CaCl and also got monsters. Scott (1906) using K Cl, $KNO_3$ and $CaCl_2$ got monsters and no cellulation. Allyn (1912) obtained the best results with heat. He treated eggs for forty minutes at 32.5° - 34.5° C. This was a real start to the problem of how to approach normalcy in the results. Finally, Just 1915 working with the eggs of Nereis got

swimming embryos by drying the female on filter paper, then removing the eggs. The eggs were then put into a watch glass and transferred from there to sea water at $34^{o}$-$35^{o}$ C. The eggs secreted a jelly and ninety to one hundred percent of them cleaved. Twenty percent of these reached the swimming embryo stage. The eggs are disposed toward cleavage by this drying. The reason that I have listed these scientists' names, experiments and dates is to show that while it is a matter of seconds to relate the results and state facts, it takes many years of experimental work, a life-time often, many failures and a great expenditure of energy to obtain positive results.

Loeb seems to have obtained fairly good results using the eggs of Polynoe. He noticed that in fertilization the sperm enters the egg while it is yet immature and that it is dependent on the formation of a fertilization membrane to develop into a larva. He treated eggs with weak solutions of two drops of saponin or salonin - 5cc. of sea water. This treatment did not cause membrane formation. Later, when eggs were subject to a hypertonic solution of 50 cc. sea water + 8cc. 2 1/2 m NaCl, they all formed membranes. The treatment with the hypertonic solution causes more eggs to develop than if it is not used. Within eight hours after treatment, the eggs had reached the trochophere stage. If the alkalinity of the sea water in which eggs are placed is increased, a large percentage will develop

without segmenting. The best reaults were obtained by using weak bases and acids especially butylamine and benzylamine. Next in efficiency were $NH_4$ OH and triethylamine. The weakest effects were gained with strong bases Na OH and tetraethylammoniumhydroxide. Those eggs treated with NaOH reached the swimming stage much later than those treated with an amine. Eggs treated with an amine, butylamine, segmented much more slowly, though almost normally, than those fertilized with sperm. Eggs exposed to alkaline sea water, though not hypertonic, developed to the larval stage without segmentation, but more slowly than those which segmented when treated with hypertonic sea water. These last developed more slowly than those fertilized with sperm. The hypertonic solution remedies the abnormal condition of non-segmentation in the larvae. Loeb found that eggs segmented normally when they were put into a solution for one and a half to two and a half minutes of 25 cc. 3/8 m S + 25 cc. $\frac{m}{2}$ Na Cl + KCl + $CaCl_2$. After this they were treated for ten minutes with serum diluted by its own volume $\frac{m}{2}$ Ringer solution. The larvae thus obtained were not quite normal, in that they tended to stick to the glass dish in which they were contained and their cleavage cells tended to fall apart easily.

Working with Fucus vesiculosis, Loeb found that eggs subjected to a solution of 50 cc. sea water + 3 cc. of

0.1 m asetic or butyric or other fatty acids formed membranes. Those eggs not fertilized or treated with a solution as above shown degenerate and cytolosis sets in. The eggs treated with the above solution developed normally.

Artificial parthenogenesis in the frog eggs is quite another matter than with sea urchin eggs, molluscs eggs and those of annellids. The only results obtained by treating frogs eggs with solutions was to produce imperfect cleavage furrows.

Guyer(1907) punctured frogs eggs with a capillary tube and injected into them blood and lymph. He obtained good cleavage and young embryos, but peculiarly enough and quite mistakenly he believed that the injected leucocytes were growing in the egg and causing it to grow.

Bataillon (1910) did a great deal of work with this method but produced only a few larvae. In 1911-1914 he came to the conclusion that only puncturing the egg with a fine glass or platinum tube was sufficient to start development and that further treatment was necessary to carry the egg to the larval stage. He believed that this second substance used in the treatment was something mysterious found in the blood. His work, however, is clouded with doubt and uncertainty as to what this substance is.

It was not until Herlant (1912-1913) performed experiments on frogs eggs with blood present at this puncturing

and without it that the matter was more or less cleared up. Eggs free from lymph were punctured with a glass tube and a fertilization membrane was formed. The eggs rotate in respect to gravity with the black hemisphere upward. The second polar body is given off and the egg nucleus which has formed migrates to the center of the egg. Forty minutes later rays appear in the protoplasm, centering near the nucleus. They become more and more pronounced until the interior of the egg is occupied by a dense ring pierced by rays. After about seventy-five minutes the nucleus comes to lie at or near the center of the ring. The eggs die in about five hours after a few mitotic divisions have taken place.

In the case of eggs punctured when blood is present cytasters form and develop at the inner side of the puncture after about an hour and a half. Rays are formed around the nucleus. After two and three-quarter hours the monaster starts to fade out and several small asters may be seen. These cytasters encroach on the monaster and seem to push it to one side where it disappears. Herland says that the cyasters bring about the division of the egg and that it is the irregular development of the asters that injures the cleavages and development of the egg into a normal larva. These asters impair later development and the eggs may fail to divide or may cleave irregularly.

Loeb and Bancroft (1913) obtained one frog and one tadpole from treating eggs of the Southern leopard frog parthenogenetically. Seven hundred eggs were thus treated. These two died and their gonads contained eggs, but there is an intermediate stage in the development of these frogs during which time their gonads contain eggs. This results from normal fertilization. Later these eggs disintegrate in the case of the male frog. There is, therefore, no reason to believe that these frogs were female due to their artificially parthenogenetic treatment. In 1919 Loeb raised twenty-one frogs to metamorphosis. Parmenter received and studied some of this material. He reports that both sexes have the diploid number of chromosomes. It seems that the chromosome number varies in the same and different individuals for in five clear cases some had twenty-six chromosomes while two had twenty-seven.

Bataillon (1904, 1910, 1911) found that in tadpoles seventeen hours old, there is the haploid number of chromosomes. Dehorne (1910) found that haploid condition in still younger stages. Brochet (1911) observed that eighteen day old tadpoles were diploid. Levy (1913) found that haploid condition in swimming tadpoles

and later in 1920 he found that in abnormal tadpoles there were from eight to twenty-four chromosomes in the epithelial cells of the tail. Hovasse (1920, 1922) observed that in the young stages sixty-five tadpoles had the haploid number, seventy-five the diploid, fourteen had an aberrant number, and the number varied often in these in the same individual. He noticed that if the nucleus divides before the first cleavage takes place the result is a haploid individual. If the chromosomes are doubled before the first cleavage the resulting tadpole is diploid. Irregularities in the first or later divisions, he believes, may account for those that are aberrant in the number of their chromosomes. Probably all but the diploid individuals die sooner or later in their development.

Another very interesting subject closely allied to the field with which we have been working is that of combining artificial parthenogenesis with that of normal fertilization. This paper would not be complete without touching upon it briefly.

Loeb used the sperm of Asterias to fertilize the eggs of S. purpuratus. The eggs thus treated developed quite normally until they reached the gastrula stage; then large numbers of them died. The few eggs that reached the pluteus stage were thoroughly characteristic

of S. purpuratus. This shows that maternal plutei will result from cross fertilization and that the sperm did not carry hereditary factors to the egg. Furthermore, these cases of cross fertilization or heterogeneous hibridization are nothing more than cases of artificial parthenogenesis.

Hertwig subjected frog sperm to radium bromide, and found that they would not fuse with the egg nucleus. Nevertheless, apparently normal tadpoles which lived for four or five weeks were produced.

Experiments have been completed with sperm to try and find out if sperm could produce an embryo. It was observed that spermatozoa form a nucleus in the white of an egg with the yolk present, but no mitosis was observed, so we cannot yet say that a sperm can or can not form an embryo.

Herbst (1906) placed the eggs of Sphaerechinus in 50 cc. sea water + 3 cc. 1/10 N NaOH and later into normal sea water where they were fertilized with the sperm of Strongylocentrotus. The resulting plutei were more like the maternal type than like the hybrid plutei from untreated eggs. This parthenogenetic treatment doubles the number of chromosomes which are tripled on introduction of the sperm. The results were

irregular. In maternal eggs the diploid nucleus is twice as large as the haploid are. If one aster forms before division, the diploid number of chromosomes is present. If two asters are present before division, the triploid number is the result. Plutei may be maternal on one side with small nuclei, and may be paternal on the other with skeletal correspondences. Some are purely maternal, others purely paternal and still others are mixed or thelykargotic.

Sperm chromosomes seem to play a very unimportant part in this type of fertilization, though the asters which form for the first division arise near the sperm nucleus and seem to be under its control. Sperm chromatin appears to be less developed than egg chromatin, and sometimes lags behind the egg chromatin in resolving itself into its constituent chromosomes. It is questionable, however, whether, if only a few sperm chromosomes become incorporated in the egg, normal development can take place.

Hinderer (1914) placed eggs of Sphaerechinus in a solution of 20 cc. $CO_2$ sea water + 30 cc. sea water and fertilized them after fifteen hours with the sperm of Strongylocentrotus. Some had double or treble nuclei, while others were normal with eighteen or twenty chromosomes. This shows that the number of egg chromosomes increased when they were treated for parthenogenetic development. Male chromosomes may be lost, but when they remain they influence the plutei.

(SUMMARY)

Landauer (1922) treated the eggs of Sphaerechinus granularis with a solution of 100 cm. sea water + 2 cm. 1 1/10 n $NH_3$ for from fifteen minutes to an hour and a half and then fertilized them with Strongylocentrotus sperm. He found that the male chromosomes were not left out of the polar spindle and divided regularly at each division of the egg. Also he observed that the skeleton of the triploid and tetraploid plutei is more like that of the pure Sphaerechinus than like that of the hybrid which stands between the parental types. "This result", Morgan[1] says, "may be significant if, as seems to be the case, the block to development may be removed from the nucleus without producing cortical changes. Since ammonia solution does not lead to complete parthenogenetic development, the result may also be interpreted to mean that cortical changes have been started, sufficient to remove the block inhibiting the division of the chromosomes (resolution of the egg nucleus) but without altering the surface to the extent of interfering with subsequent fertilization."

In concluding this paper I should like to quote Dr. Morgan onee more in regard to what has been accomplished in the field of artificial parthenogenesis and what we may hope to expect from it in the future. He[2] says: "The extensive literature of artificial parthenogenesis shows

[1]T. H. Morgan, Experimental Embryology, page 593

[2]T. H. Morgan, Experimental Embryology, pages 580-581.

only too clearly how futile it is at present to speculate as to the chemical reaction that starts the egg on its course of development. Whether the artificial agent causes a change only begins there, cannot be positively asserted, while it is not very enlightening to speak of this effect as the removal of a block that holds the egg in check. Such a view has the merit, at least, of throwing the emphasis back on the egg itself as the principal actor in the event, but unless the nature of the block can be defined, the statement is only a figure of speech. The initiation of development has also been said to be due to a stimulus, but unless the nature of the stimulus can be defined the comparison has little or no value. A change in surface tension has also been suggested, but how such a change could start development is as difficult to explain at present as the observations themselves. Loeb has laid much emphasis on the cytolosis of the surface layer, but the nature of the special kind of cytolosis and its chemical equivalent is left unexplained. It does not seem probable that the instantaneous effect of the penetration of the tip of the sperm could cause such an effect in normal fertilization, even granting that the influence starts at the penetration point and passes around (or through?) the egg. Until more is known of the chemico-physical changes that take place both in normal and in artificial fertili-

zation, the suggestions that have been made can not be considered more than speculative. The fact that unfertilized eggs may be induced to develop into embryos by artificial agents of the most diverse kind, rather than the hypothesis to account for the change, is the outstanding feature of all this work."

author, the suggestions that have been made can not be considered more than speculation. The fact that investigators may be induced to develop into [illegible] additional aspects of the most attractive field, rather than the hypothesis to account for the disease, is the outstanding feature of all this work."

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